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CLINICAL RESEARCH
Acute coronary syndromes

Protein-based cardiogenic shock patient classifier

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Aims Cardiogenic shock (CS) is associated with high short-term mortality and a precise CS risk stratification could guide interventions to improve patient outcome. Here, we developed a circulating protein-based score to predict short-term mortality risk among patients with CS.

Methods and results Mass spectrometry analysis of 2654 proteins was used for screening in the Barcelona discovery cohort ($n = 48$). Targeted quantitative proteomics analyses ($n = 51$ proteins) were used in the independent CardShock cohort ($n = 97$) to derive and cross-validate the protein classifier. The combination of four circulating proteins (Cardiogenic Shock 4 proteins—CS4P), discriminated patients with low and high 90-day risk of mortality. CS4P comprises the abundances of liver-type fatty acid-binding protein, beta-2-microglobulin, fructose-bisphosphate aldolase B, and SerpinG1. Within the CardShock cohort used for internal validation, the C-statistic was 0.78 for the CardShock risk score, 0.83 for the CS4P model, and 0.84 ($P = 0.033$ vs. CardShock risk score) for the combination of CardShock risk score with the CS4P model. The CardShock risk score with the CS4P model showed a marked benefit in patient reclassification, with a net reclassification improvement (NRI) of 0.49 ($P = 0.020$) compared with CardShock risk score. Similar reclassification metrics were observed in the IABP-SHOCK II risk score combined with CS4P (NRI =0.57; $P = 0.032$). The CS4P patient classification power was confirmed by enzyme-linked immunosorbent assay (ELISA).

Conclusion A new protein-based CS patient classifier, the CS4P, was developed for short-term mortality risk stratification. CS4P improved predictive metrics in combination with contemporary risk scores, which may guide clinicians in selecting patients for advanced therapies.

Keywords Cardiogenic shock • Proteome • Mortality • 90 days

Introduction

Well into the 21st century, cardiogenic shock (CS) remains associated with unacceptable high mortality, substantial morbidity, and resource utilization.[1] Despite widespread use of early coronary reperfusion, CS prevalence remains unaltered in ST-segment elevation myocardial infarction (STEMI) and it is the leading cause of in-hospital death.[2–4]

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Contemporary risk scores are available, including the CardShock and IABP-SHOCK II risk scores, which mostly rely on classical clinical acumen and conventional laboratory variables, i.e. lactate and glucose.[5,6] However, more accurate risk stratification strategies are needed for early determination of when palliative or invasive therapies are likely most appropriate or futile in CS.[7]

Accumulating evidence indicates that CS is not only a pump failure problem but is rather a systemic inflammatory status within the context of multiorgan failure.[8–11] Therefore, comprehensive proteomics may enable the discovery of novel protein biomarkers that can be used to acquire pathophysiological knowledge, improve risk stratification accuracy, and identify therapeutic targets.[12]

In the present study, we used quantitative proteomics to identify and cross-validate a protein-based biomarker combination (the Cardiogenic Shock 4 Proteins—CS4P) as a new CS risk assessment score model for short-term mortality. We next tested the CS4P into enzyme-linked immunosorbent assays (ELISA) to favour its prompt translation into routine clinical practice (*Take home figure*).

Methods

Patient cohorts

The Barcelona discovery cohort is a prospective single-centre all-comers study between March 2011 and March 2015, including consecutive patients with STEMI complicated with CS during the first 24 h of evolution. ST-segment elevation myocardial infarction was defined according to the Third Universal Definition of Myocardial Infarction.[13] Patient management was determined by the physicians, following guideline recommendations.[14,15] Two plasma samples were obtained (admission and at 24 h) from every patient ($n = 48$) by venipuncture and stored at -80°C (Supplementary material online, *Figure S1*).

The CardShock cohort is a European prospective, multicentre, multinational study on CS between October 2010 and December 2012, including patients within 6 h from identification of CS with both acute coronary syndrome (ACS) and non-ACS aetiologies.[5] Cohort clinical characteristics and inclusion and exclusion criteria are reported elsewhere.[5] For the present study, only one plasma sample withdrawn within 24 h of admission, immediately frozen and stored at –80°C, was used ($n = 97$) (Supplementary material online, *Figure S1*). No significant differences were observed between CardShock included and non-included patients (absence of biorepository; Supplementary material online, *Table ST1*).

Inclusion and exclusion criteria were similar in both studies.[5] Briefly, inclusion criteria required systolic blood pressure (SBP) to be <90 mmHg for 30 min (or there to be a need for vasopressor therapy to maintain SBP >90 mmHg) and signs of hypoperfusion (altered mental status, cold periphery, oliguria, or blood lactate >2 mmol/L). Exclusion criteria were shock caused by ongoing haemodynamically significant arrhythmias or after cardiac or non-cardiac surgery.

During follow-up, vital status was determined by direct contact with the patients or their next of kin, or from population and hospital registers. The clinical endpoint was 90-day mortality.

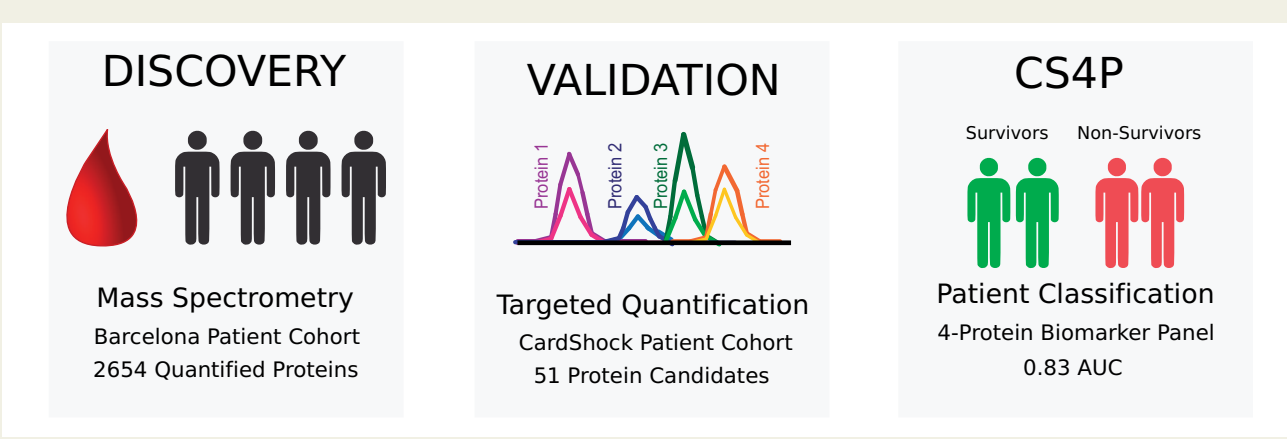
Both cohorts were approved by local ethics committees at the participating centres, and studies were conducted in accordance with the Declaration of Helsinki. Written consent was obtained from the patients or their next of kin.

Biomarker discovery by screening proteomics

Quantitative proteomics analysis was performed using mass spectrometry (nLC-MS/MS) to identify potential protein biomarker candidates among those proteins differing in abundance between 90-day survivors and non-survivors. Plasma samples (admission and at 24 h) from 48 patients of the Barcelona cohort (21 non-survivors and 27 survivors at 90 days) were trypsin digested to peptides and analysed using label-free screening proteomics (nLC-MS/MS). Details on sample processing, chromatography separation, mass spectrometry acquisition, and data analysis are provided in Supplementary material online, *Methods*. The discovery proteomics data have been deposited to the ProteomeXchange Consortium with the dataset identifier PXD011614.

Biomarker cross-validation by targeted proteomics

Candidate biomarker proteins, identified in the discovery phase, were evaluated in terms of classification power in the CardShock



Take home figure General workflow of the study.

cohort with targeted proteomics quantification using parallel reaction monitoring (PRM). Plasma samples corresponding to 97 patients from the CardShock cohort (36 non-survivors and 61 survivors at 90 days), were trypsin digested and analysed using targeted nLC-PRM and isotopically labelled standard peptides as internal references. Fragment ion chromatographic traces for precursor peptides were evaluated, logarithmic transformed, and normalized using the internal reference peptides. Protein abundances were estimated, and protein relative quantification was assessed between survivors and non-survivors. Assay variability was calculated with seven injections of a mixture of the isotopically labelled reference peptides expanding 3 weeks of the acquisition of the patient samples. Details on sample processing and PRM are presented in Supplementary material online, *Methods*. The targeted proteomics data have been deposited to the ProteomeXchange Consortium with the dataset identifier PXD011593.

Enzyme-linked immunosorbent assays of validated proteins

Four commercially available ELISA kits were used, following the instructions of the manufacturer, for each validated protein in all patients from the CardShock cohort. Details on each ELISA assay are presented in Supplementary material online, *Methods*.

Statistical analyses

Clinical variables are presented as number (*n*) and percentage (%) for categorical variables, mean and standard deviation for normally distributed variables, or median and interquartile range (IQR) for skewed variables. Comparisons between groups were performed using the χ^2 test, the Student's *t*-test, or Mann–Whitney *U* test as appropriate.

Protein abundance estimates and relative protein quantification between groups (survivors vs. non-survivors) from proteomics data were performed with the software packages Skyline 3.7[16] and MSstats 3.8.2 (details in Supplementary material online, *Methods*). The best protein combinations for classifying 90-day mortality risk in patients with CS were challenged within the CardShock cohort, which was divided into a training subset (two of three patients) and a validation subset (one of three patients). Within the training set the abundance of each protein was fitted in a logistic regression model between survivors and non-survivors, and the classification ability of each protein was evaluated by the area under the curve (AUC) of a receiver operating characteristic. The protein with the highest AUC was selected as the first classifier. Most discriminative proteins were repeatedly added to the classifier as long as their combination resulted in an increase of AUC value higher than 0.01. The obtained protein combination in the training subset was then fitted in a logistic regression model and was applied on the validation subset. The described procedure was repeated 500 times to assess the reproducibility of classification ability, and the final consensus model was comprised of the combination of proteins which were selected the most in the 500 repeats.[17]

The CardShock risk score, used as baseline model, includes age >75 years, confusion at presentation, previous myocardial infarction or coronary artery bypass grafting (CABG), ACS aetiology, left ventricular ejection fraction <40%, blood lactate, and estimated glomerular filtration rate by the Chronic Kidney Disease Epidemiology Collaboration formula ($eGFR_{CKD\text{-}EPI}$). Model calibrations were calculated using the Hosmer–Lemeshow test, and patient discrimination and reclassification were evaluated using the Harrell C-statistic (AUC) and continuous net reclassification improvement (cNRI). In the cNRI, we calculated the proportion of non-survivors that increased their risk probability, and the survivors that decreased their risk probability by the updated model compared with the baseline model. Confidence intervals for the C-statistic and the NRI were obtained by 1000-fold bootstrap resampling. Differences were considered statistically significant at $P < 0.05$. Analyses were performed using STATA V.13.0 (StataCorp, College Station, TX, USA), PredictABEL R package v1.2,[18] and SPSS V.20.0 (IBM Corp, Armonk, NY, USA).

Results

Discovery of protein biomarker candidates (Barcelona cohort)

Table 1 shows the clinical, biochemical, and follow-up data from the Barcelona discovery cohort. The mean age was 69 ± 13 years, 35% were women, and 94% were treated with primary percutaneous coronary intervention. Ninety-day mortality was 45.8%.

A total of 2654 proteins were identified by discovery mass spectrometry-based proteomics, of which 488 were present in over 30% of the patients (Supplementary material online, *Table ST2*). After protein relative quantification among the different variables: patient outcome (survivors and non-survivors) and sampling time (admission, 24 h). A total of 51 proteins were considered for analysis in the independent CardShock cohort (*Table 2*). Briefly, 32 of these proteins were directly selected from the derivation cohort as they differed between survivors and non-survivors (*P* value < 0.05) in one of the following manners: (i) proteins exhibited differential abundance between survivors and non-survivors either at admission or at 24 h; and (ii) proteins changed over time (admission vs. 24 h) exclusively for survivors or non-survivors. Additionally, 19 proteins were also included in the study based on previous knowledge and clinical relevance.

Targeted proteomics cross-validation of circulating biomarker candidates (CardShock cohort)

The prediction power of short-term mortality among CS patients of the 51 selected proteins was further assessed in the CardShock cohort using targeted proteomics quantification by PRM. *Table 1* shows the characteristics of the CardShock independent cohort. The mean age was 66 ± 14 years, 25% were women, and 90-day mortality was 37.1%. The most common cause of CS was ACS (71%) and mainly driven by STEMI (52%). Compared to the Barcelona Cohort, CardShock patients exhibited higher rates of prior heart failure (HF) and myocardial infarction, and lower blood pressure, although a more favourable biochemical profile, with higher haemoglobin and lower creatinine, lactate, and glucose levels (*Table 1*). Samples were harvested within a range of 21–33 h from detection of shock.

Table 1 Comparison of baseline characteristics, clinical presentation, management, analytical parameters, and outcome between the Barcelona and CardShock cohorts

Characteristics	Barcelona cohort (*n* = 48)	CardShock cohort (*n* = 97)	*P* value
Age (years)	68.8 (13.2)	65.7 (14.0)	0.204
Women, *n* (%)	17 (35.4)	24 (24.7)	0.179
Medical history, *n* (%)			
Hypertension	30 (62.5)	57 (58.8)	0.666
Diabetes	20 (41.7)	29 (29.9)	0.159
Stroke/TIA	6 (12.5)	11 (11.3)	0.838
Prior heart failure	5 (10.4)	22 (22.7)	0.074
Coronary artery disease	12 (25.0)	34 (35.1)	0.221
Previous myocardial infarction	4 (8.3)	24 (24.7)	0.018
Prior PCI	5 (10.4)	16 (16.5)	0.328
Prior CABG	1 (2.1)	6 (6.2)	0.278
Clinical presentation			
Acute coronary syndrome, *n* (%)	48 (100)	69 (71.1)	<0.001
STEMI, *n* (%)	48 (100)	50 (51.5)	<0.001
Resuscitated from cardiac arrest, *n* (%)	15 (31.3)	20 (20.6)	0.159
Mean blood pressure (mmHg)	69 ± 20	57 ± 10	<0.001
Heart rate (b.p.m.)	85 ± 29	92 ± 28	0.167
LVEF (%)	39 ± 13	34 ± 15	0.057
Management, *n* (%)			
Coronary angiography	46 (95.8)	73 (75.3)	0.002
PCI	45 (93.8)	58 (59.8)	<0.001
TIMI flow after PCI			0.104
0	0 (0)	3 (5.1)	
1	0 (0)	4 (6.8)	
2	4 (10.5)	10 (16.9)	
3	34 (89.5)	42 (71.2)	
CABG	0 (0)	5 (5.3)	0.104
IABP	27 (56.3)	45 (46.4)	0.264
Biochemistry at admission			
Haemoglobin (g/L)	118 ± 24	128 ± 25	0.024
Creatinine (mg/dL), median (IQR)	1.6 (1.3–1.9)	1.2 (0.9–1.6)	<0.001
$eGFR_{CKD\text{-}EPI}$ (mL/min/1.73 m^2)	42 ± 15	63 ± 29	<0.001
Arterial blood lactate (mmol/L), median (IQR)	6.3 (4.6–15.0)	2.5 (1.8–5.6)	0.003
Lactate >5 mmol/L, *n* (%)	5 (71.4)	26 (26.8)	0.013
Glucose (mmol/L)	16.9 ± 7.3	11.9 ± 6.0	<0.001
Glucose >10.6 mmol/L, *n* (%)	38 (84.4)	46 (48.4)	<0.001
hsTnT (pg/mL), median (IQR)	2116 (869–7690)	1568 (277–3920)	0.068
NT-proBNP (pg/mL), median (IQR)	1945 (558–6984)	3385 (687–9716)	0.142
90-Day mortality, *n* (%)	22 (45.8)	36 (37.1)	0.313

CABG, coronary artery bypass grafting; $eGFR_{CKD\text{-}EPI}$, estimated glomerular filtration rate by the Chronic Kidney Disease Epidemiology Collaboration formula; hsTnT, high-sensitivity troponin T; IABP, intra-aortic balloon pump; LVEF, left ventricular ejection fraction on admission; NT-proBNP, *N*-terminal pro-B-type natriuretic peptide; PCI, percutaneous coronary intervention; STEMI, ST-elevation myocardial infarction; TIA, transient ischaemic attack; TIMI, thrombolysis in myocardial infarction.

High-quality mass spectrometry chromatographic profiles were obtained for 26 targeted proteins (*Figure 1*) and compared to the corresponding internal references for relative protein quantification (Supplementary material online, *Tables ST3* and *ST4* and *Figures S2* and *S3*). The assay variability was calculated as coefficient of variation (CV) and 96.2% of the targeted peptides exhibited a CV <20% being the highest value 25% (Supplementary material online, *Figure S4*). The best protein combinations for classifying 90-day survivors and non-survivors in CS patients were identified by performing a predictor selection combined with cross-validation as described in the Methods section. This evaluation resulted in the identification of a 4-protein combination—the CS4P—with proteins liver-type fatty acid-binding protein (L-FABP, P07148), beta-2-microglobulin (B2MG, P61769), fructose-bisphosphate aldolase B (ALDOB, P05062), and SerpinG1

Table 2 List of the 51 proteins selected from screening proteomics for targeted cross-validation

Accession	Description
Q8N7J2	APC membrane recruitment protein 2 OS = Homo sapiens GN = AMER2 PE = 1 SV = 3
Q5VTU8	ATP synthase subunit epsilon-like protein, mitochondrial OS = Homo sapiens GN = ATP5EP2 PE = 3 SV = 1
P61769	Beta-2-microglobulin OS = Homo sapiens GN = B2M PE = 1 SV = 1
Q9NYQ6	Cadherin EGF LAG seven-pass G-type receptor 1 OS = Homo sapiens GN = CELSR1 PE = 1 SV = 1
P43121	Cell surface glycoprotein MUC18 OS = Homo sapiens GN = MCAM PE = 1 SV = 2
Q92496	Complement factor H-related protein 4 OS = Homo sapiens GN = CFHR4 PE = 1 SV = 3
O14733	Dual specificity mitogen-activated protein kinase kinase 7 OS = Homo sapiens GN = MAP2K7 PE = 1 SV = 2
O60941	Dystrobrevin beta OS = Homo sapiens GN = DTNB PE = 1 SV = 1
P07148	Fatty acid-binding protein, liver OS = Homo sapiens GN = FABP1 PE = 1 SV = 1
P05062	Fructose-bisphosphate aldolase B OS = Homo sapiens GN = ALDOB PE = 1 SV = 2
A6NHX0	GATS-like protein 2 OS = Homo sapiens GN = GATSL2 PE = 2 SV = 3
P00739	Haptoglobin-related protein OS = Homo sapiens GN = HPR PE = 2 SV = 2
P18065	Insulin-like growth factor-binding protein 2 OS = Homo sapiens GN = IGFBP2 PE = 1 SV = 2
Q13094	Lymphocyte cytosolic protein 2 OS = Homo sapiens GN = LCP2 PE = 1 SV = 1
Q14833	Metabotropic glutamate receptor 4 OS = Homo sapiens GN = GRM4 PE=2 SV = 1
Q5T6S3	PHD finger protein 19 OS = Homo sapiens GN = PHF19 PE = 1 SV = 1
Q9Y285	Phenylalanine—tRNA ligase alpha subunit OS = Homo sapiens GN = FARSA PE = 1 SV = 3
O60879	Protein diaphanous homologue 2 OS = Homo sapiens GN = DIAPH2 PE = 1 SV = 1
P78504	Protein jagged-1 OS = Homo sapiens GN = JAG1 PE = 1 SV = 3
P05109	Protein S100-A8 OS = Homo sapiens GN = S100A8 PE = 1 SV = 1
P20848	Putative alpha-1-antitrypsin-related protein OS = Homo sapiens GN = SERPINA2 PE = 1 SV = 1
Q2PPJ7	Ral GTPase-activating protein subunit alpha-2 OS = Homo sapiens GN = RALGAPA2 PE = 1 SV = 2
P02753	Retinol-binding protein 4 OS = Homo sapiens GN = RBP4 PE = 1 SV = 3
Q6XE24	RNA-binding motif, single-stranded-interacting protein 3 OS = Homo sapiens GN = RBMS3 PE = 1 SV = 1
P49908	Selenoprotein P OS = Homo sapiens GN = SEPP1 PE = 1 SV = 3
Q99835	Smoothened homologue OS = Homo sapiens GN = SMO PE = 1 SV = 1
P50225	Sulfotransferase 1A1 OS = Homo sapiens GN = SULT1A1 PE = 1 SV = 3
Q5SNT2	Transmembrane protein 201 OS = Homo sapiens GN = TMEM201 PE = 1 SV = 1
Q8N655	Uncharacterized protein C10orf12 OS = Homo sapiens GN = C10orf12 PE = 1 SV = 1
Q9Y4I1	Unconventional myosin-Va OS = Homo sapiens GN = MYO5A PE = 1 SV = 2
Q9NZ43	Vesicle transport protein USE1 OS = Homo sapiens GN = USE1 PE = 1 SV = 2
Q15878	Voltage-dependent R-type calcium channel subunit alpha-1E OS = Homo sapiens GN = CACNA1E PE = 1 SV = 3
P01011	Alpha-1-antichymotrypsin OS = Homo sapiens GN = SERPINA3 PE = 1 SV = 2
P01019	Angiotensinogen OS = Homo sapiens GN = AGT PE = 1 SV = 1
P02741	C-reactive protein OS = Homo sapiens GN = CRP PE = 1 SV = 1
P02671	Fibrinogen alpha chain OS = Homo sapiens GN = FGA PE = 1 SV = 2
P02675	Fibrinogen beta chain OS = Homo sapiens GN = FGB PE = 1 SV = 2
P02679	Fibrinogen gamma chain OS = Homo sapiens GN = FGG PE = 1 SV = 3
P05546	Heparin cofactor 2 OS = Homo sapiens GN = SERPIND1 PE = 1 SV = 3
P05121	Plasminogen activator inhibitor 1 OS = Homo sapiens OX = 9606 GN = SERPINE1 PE = 1 SV = 1
P00747	Plasminogen OS = Homo sapiens GN = PLG PE = 1 SV = 2
P00488	Coagulation factor XIII A chain OS = Homo sapiens GN = F13A1 PE = 1 SV = 4
Q01638	Interleukin-1 receptor-like 1 OS = Homo sapiens OX = 9606 GN = IL1RL1 PE = 1 SV = 4
P16860	Natriuretic peptides B OS = Homo sapiens OX = 9606 GN = NPPB PE = 1 SV = 1
P45379	Troponin T, cardiac muscle OS = Homo sapiens OX = 9606 GN = TNNT2 PE = 1 SV = 3
P19429	Troponin I, cardiac muscle OS = Homo sapiens OX = 9606 GN = TNNI3 PE = 1 SV = 3
P62979	Ubiquitin-40S ribosomal protein S27a OS = Homo sapiens OX = 9606 GN = RPS27A PE = 1 SV = 2
P04075	Fructose-bisphosphate aldolase A OS = Homo sapiens OX = 9606 GN = ALDOA PE = 1 SV = 2
P04040	Catalase OS = Homo sapiens OX = 9606 GN = CAT PE = 1 SV = 3
P05155	Plasma protease C1 inhibitor OS = Homo sapiens OX = 9606 GN = SERPING1 PE = 1 SV = 2
P04114	Apolipoprotein B-100 OS = Homo sapiens OX = 9606 GN = APOB PE = 1 SV = 2

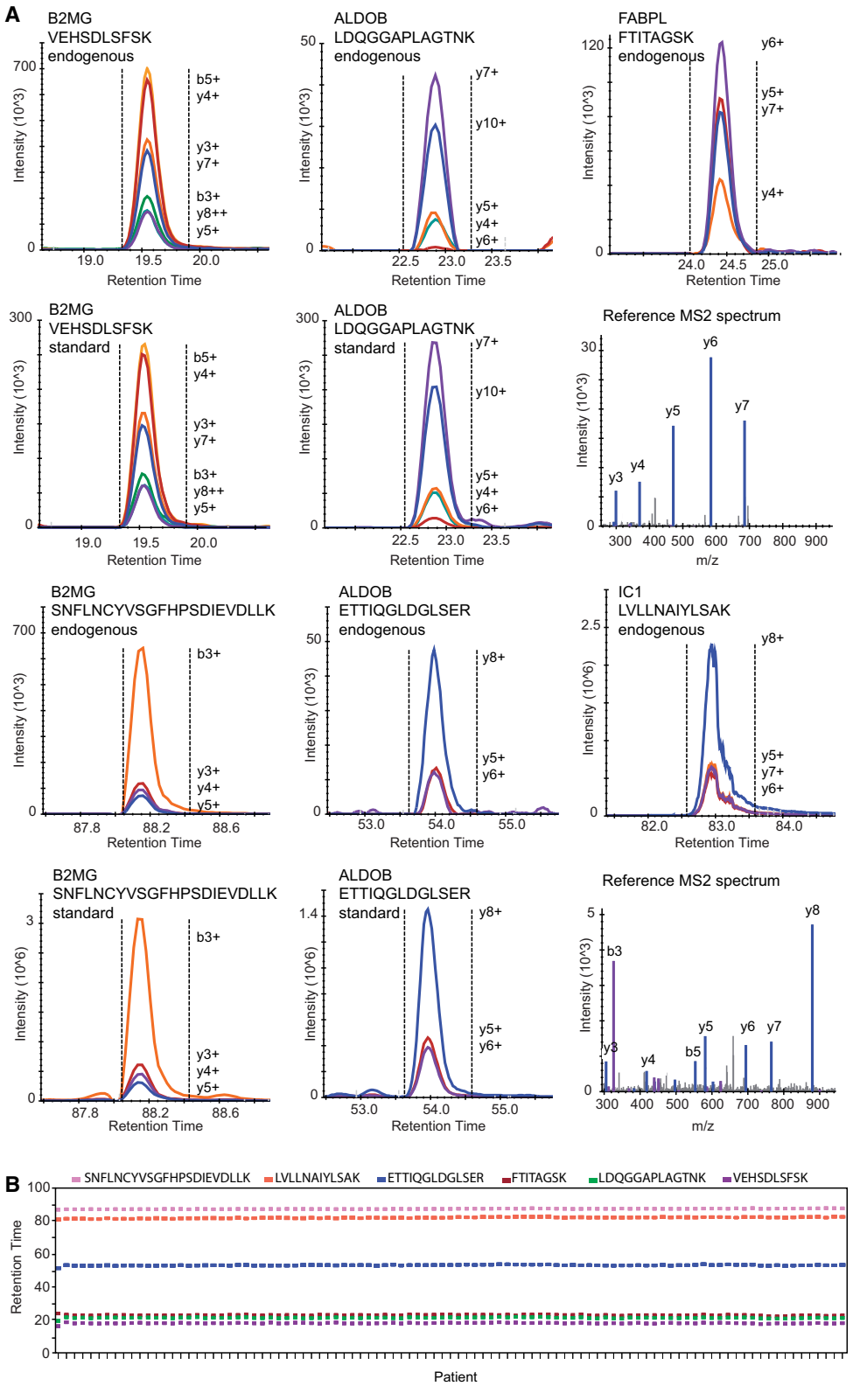


Figure 1 Targeted proteomics results. (*A*) Targeted mass spectrometry chromatographic profiles corresponding to the endogenous peptides and their isotopically labelled internal standards (or reference MS2 spectra) of the proteins L-FABP, B2MG, ALDOB, and IC1. (*B*) Retention time drift of the endogenous peptides shown in (*A*) for all analysed patients. L-FABP, liver-type fatty acid-binding protein; ALDOB, fructose-bisphosphate aldolase B; B2MG, beta-2-microglobulin; IC1, SerpinG1.

Table 3 Comparison of model performances for predicting 90-day mortality in cardiogenic shock patients

	AUC	HL	*P*-value	NRI	Threshold (Youden's J)	Sensitivity	Specificity
CardShock	0.78 (0.69–0.87)	3.29	0.914	–	0.25	0.91	0.54
IABP-Shock II[a]	0.78 (0.66–0.90)	6.64	0.576	–	0.26	0.85	0.58
CS4P	0.83 (0.74–0.89)	13.26	0.103	–	0.36	0.83	0.75
CardShock + CS4P	0.84 (0.76–0.93)	7.25	0.509	0.49	0.47	0.77	0.84
IABP-Shock II + CS4P	0.80 (0.67–0.92)	3.44	0.904	0.57	0.35	0.85	0.68

CardShock includes age >75 years, confusion at presentation, previous myocardial infarction, or CABG, ACS aetiology, LVEF <40%, blood lactate, and $eGFR_{CKD\text{-}EPI}$. CS4P score includes liver-type fatty acid-binding protein, fructose-bisphosphate aldolase B, beta-2-microglobulin, and SerpinG1. CardShock + CS4P includes CardShock plus CS4P score.
AUC, area under the curve; HL, Hosmer–Lemeshow; NRI, net reclassification improvement.
[a]IABP-SHOCK II scores ($n = 55$) were calculated from the CardShock database, as previously reported.[6]

(IC1, P05155), as the best protein classifier to identify short-term mortality risk with an AUC of 0.83 (95% CI 0.74–0.89) (*Table 3* and *Figure 2*).

An additional model was constructed by combining the CardShock risk score with the new CS4P model (CardShock + CS4P). The CardShock + CS4P significantly improved the C-statistics for mortality prediction compared with the CardShock risk score alone (AUC 0.84 vs. 0.78, $P = 0.033$; *Table 3* and *Figure 2*). Furthermore, the CardShock + CS4P showed a marked benefit in patient reclassification, with an NRI of 0.49 ($P = 0.020$) (*Table 3*; Supplementary material online, *Figures S5* and *S6*). Overall, CardShock + CS4P resulted in an improved reclassification compared to CardShock risk score. Among survivors, 64% were down-classified and 60% up-classified using the combined CardShock + CS4P.

Baseline and 24 h samples of the Barcelona cohort were analysed in four temporal strata relative to sample collection (00:00–6:00 h, 6:00–12:00 h, 12:00–18:00 h, and 18:00–00:00 h). Samples were drawn in the same temporal distribution among survivors and non-survivors and abundance of the CSP4 proteins (measured by MS; Supplementary material online, *Table ST3*) was not significantly different in the four temporal strata in both survivors and non-survivors. Taken together, these data suggest a small relevance of the circadian pattern in the reported data.

In an exploratory analysis, we also combined the CS4P model with another contemporary risk score, the IABP-SHOCK II,[6] generating the IABP-SHOCK II + CS4P (*Table 3*). The IABP-SHOCK II + CS4P also provided better prediction metrics compared to IABP-SHOCK II, with improved C-statistics (AUC 0.80 vs. AUC 0.78) and NRI of 0.57 ($P = 0.032$).

Translation of CS4P into enzyme-linked immunosorbent assays (ELISA)

The CS4P model defined by targeted proteomics was tested by ELISA to support its prompt translation into routine clinical practice. Median (IQR) circulating concentrations of the studied proteins were L-FABP, 160 pg/mL (42–1720 pg/mL); B2MG, 482 µg/mL (276–752 µg/mL); ALDOB, 101 ng/mL (70–209 ng/mL); and IC1, 218 pg/mL (169–259 pg/mL), respectively. Circulating L-FABP (453 vs. 94 pg/mL, $P = 0.02$), B2M (709 vs. 344 µg/mL, $P < 0.001$), and ALDOB (156 vs. 84 ng/mL, $P = 0.05$) were higher in non-survivors relative to survivors. By contrast, IC1 concentration was significantly lower in non-survivors relative to survivors (205 vs. 226 pg/mL, $P = 0.02$) (*Figure 3*, Supplementary material online, *Table ST5*).

Protein concentrations of the CS4P obtained by ELISA combined with the CardShock risk score provided an AUC of 0.82 (95% CI 0.73–0.90), not significantly different than that obtained by targeted proteomics ($P = 0.123$) (Supplementary material online, *Figures S7* and *S8*).

Discussion

In the present study, we performed quantitative proteomics analyses in two independent CS cohorts for the discovery and cross-validation of CS biomarkers. First, we used discovery mass spectrometry to quantify thousands of different proteins without the need of previous knowledge, and thus identify proteins not previously associated to CS. After protein relative quantification between survivors and non-survivors the classification power of a total of 51 proteins was further evaluated in an independent cohort by targeted proteomics and cross-validation. We identified four proteins (L-FABP, B2MG, ALDOB, and IC1) for which the measured levels within 24 h of CS admission substantially improved risk prediction beyond established contemporary clinical risk scores (*Take home figure*). Based on the results, we developed a protein-based classifier—the CS4P—which was also tested by ELISA, that accurately discriminates patients according to their short-term mortality risk.

Despite the generalization of early reperfusion and modern intensive care, CS management remains challenging with mortality rates of ~40%.[19] Early and accurate risk stratification is crucial for prompt identification of the sickest patients who may benefit from advanced therapies. While clinical predictors of adverse outcome have been well-known for decades, their derivation from pre-percutaneous coronary intervention (PCI) clinical trials and a lack of external validation precluded their routine use and prompted the development of more contemporary risk classifiers. Two scores have been recently reported. The CardShock risk score[5] was developed from a large prospective multicentre European registry of unselected CS patients with a broad spectrum of aetiologies (two-thirds STEMI). The

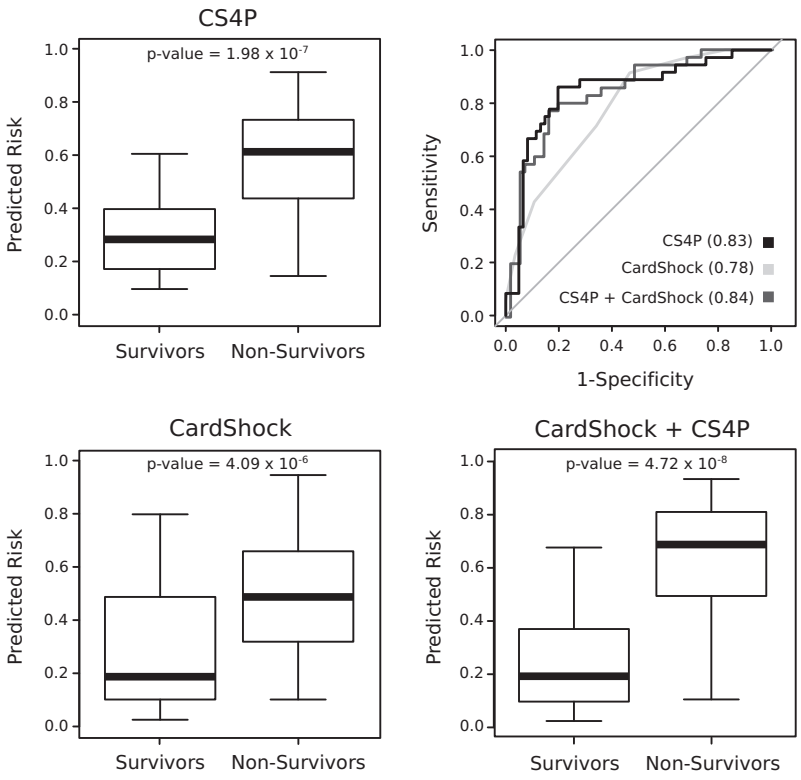


Figure 2 Improvement in discrimination for prediction of 90-day mortality risk with each model. CardShock risk score includes age >75 years, confusion at presentation, previous myocardial infarction or coronary artery bypass grafting, ACS aetiology, LVEF <40%, blood lactate, and $eGFR_{CKD\text{-}EPI}$. CS4P score includes circulating protein abundance measured by parallel reaction monitoring of the liver-type fatty acid-binding protein, the Fructose-bisphosphate aldolase B, the beta-2-microglobulin, and the SerpinG1. CardShock + CS4P includes CardShock risk score plus the CS4P score. AUC values for CS4P, CS4P + CardShock, and CardShock are 0.83, 0.84, and 0.78, respectively, and are indicated in brackets in the receiver operating characteristic plot. The Mann–Whitney test was used for statistical assessment.

IABP-SHOCK II risk score[6] was developed from IABP-SHOCK II trial participants and is specific for PCI-treated STEMI-related CS. These two scores are externally validated (of note, the IABP-SHOCK II risk score was validated by the CardShock risk score used here) and include classical clinical and biochemical variables for short-term risk stratification.

Notably, the laboratory parameters included in these risk scores are basic biochemical tests—glucose and lactate—that have been routinely used in the clinic for several decades, but also include clinical acumen parameters. More recent studies have explored cardiac and extra-cardiac predictive biomarkers in CS.[8–11,20] However, most of these studies are small or not validated by external cohorts or did not assess the incremental predictive value of such biomarkers combined with current clinical practice. Particularly, novel renal biomarkers, including cystatin C, plasma neutrophil gelatinase-associated lipocalin, and kidney injury molecule-1, have not performed better than conventional creatinine.[21] With regards to proteomics data in other cardiovascular pathology contexts, a proteomics approach was recently reported in the setting of stable coronary artery disease, in which a 9-protein risk score was reported with a C-statistic of 0.74.[22]

To our knowledge, this is the first study to employ a comprehensive quantitative proteomics approach combined with targeted proteomics to discover and validate risk assessment molecular biomarkers in CS. Our results showed that the combination of four circulating proteins (L-FABP, ALDOB, B2MG, and IC1), measured within 24 h of admission, produced the CS4P molecular classifier capable of distinguishing high-risk CS patients with a C-statistic of 0.83 for short-term mortality. This discrimination performance is at least as high as that provided by contemporary CS risk scores (CardShock and IABP-SHOCK II risk scores) and importantly, improved their performance. The translation of the CS4P at the patients' bedside should be the next logical step, but it will likely require future refinements of the ELISA immunoassays used here. At present, only B2MG has available assays validated for clinical use with FDA and CE mark. Relative to ALDOB and IC1 and L-FABP, the ELISA assays used in this study were for research use only. Nevertheless, these assays were robust and reproducible, and their validation for clinical use may be achieved in the near future. Whether a CS4P biomarker-guided approach may be of value in the setting of CS must be explored in appropriately conducted prospective randomized clinical trials.

In addition to their value as biomarkers, the four proteins identified in this study provide a better understanding of the pathophysiology of CS and multi-organ damage. Remarkably, L-FABP, ALDOB, and B2MG were significantly higher among non-survivors, while IC1 was

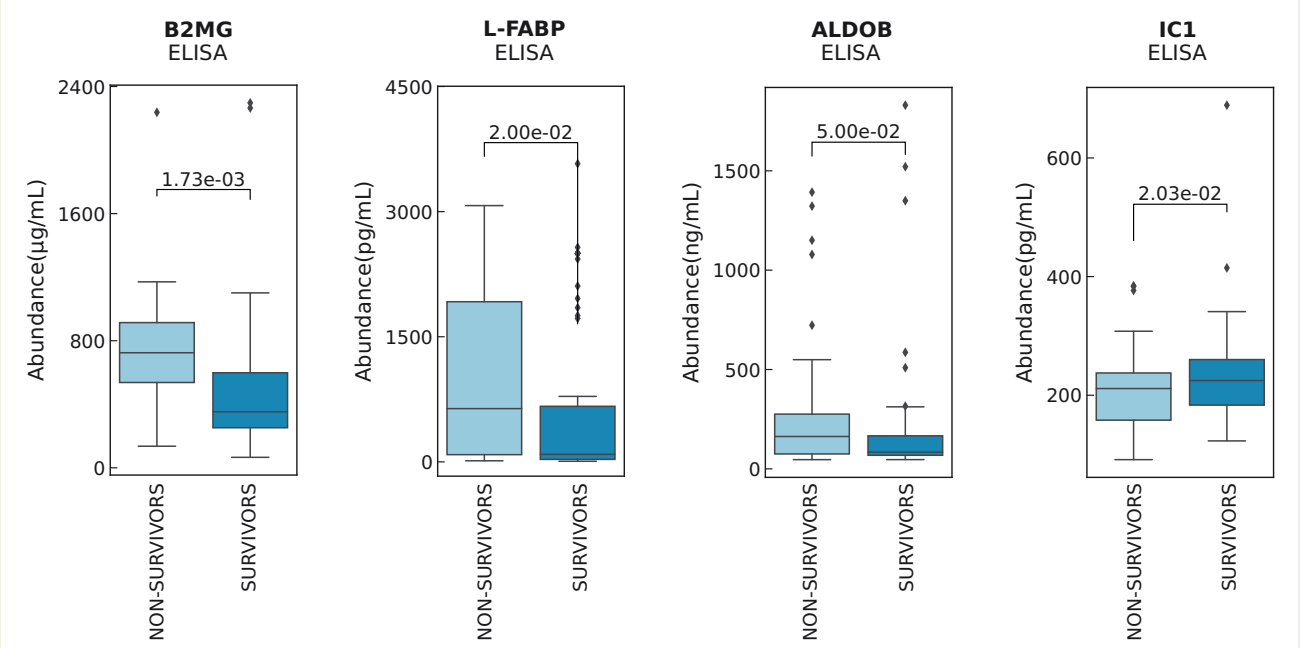


Figure 3 Box-plots relative to 90-day survivors vs. non-survivors of the four proteins of the CS4P measured by ELISA. The Student's *t*-test was used for statistical assessment. L-FABP, liver-type fatty acid-binding protein; ALDOB, fructose-bisphosphate aldolase B; B2MG, beta-2-microglobulin; IC1, SerpinG1.

significantly reduced in non-survivors relative to survivors, respectively. Liver-type fatty acid-binding protein is a cytosolic protein that participates in the intracellular transport of fatty acids to the mitochondria; binds reactive oxygen species; and is involved in intracellular signalling pathways, cell growth, and differentiation. Liver-type fatty acid-binding protein is present in the liver, intestine, stomach, lung, and kidney, and its expression is up-regulated by tissue injury. Circulating L-FABP shows an early and fleeting elevation in septic shock, which indicates liver injury and is associated with adverse outcomes.[23] Matsumori *et al.*[24] found that in patients with ACS high L-FABP may be useful in identifying high-risk patients for future cardiovascular events. In another study, Hishikari *et al.*[25] examined 281 consecutive patients with acutely decompensated HF and found that L-FABP is useful for predicting the onset of acute kidney injury. Indeed, L-FABP is involved in acute kidney tubular necrosis and chronic kidney failure.

Fructose-bisphosphate aldolase B is a key component of the glycolysis and gluconeogenesis pathways and is mainly expressed in the cortex of kidneys, liver, and small intestine Peyer's Patch. It is involved in hereditary fructose intolerance and Bardet–Biedl Syndrome. Serum ALDOB levels appear to be a useful measure of liver cell necrosis in both benign and malignant liver diseases.[26] Fructose-bisphosphate aldolase B is up-regulated during the first 4 h in a murine experimental septic shock model.[27] Fructose-bisphosphate aldolase B has also been related to obesity, liver inflammation, and hepatic fibrosis, consistent with a multiorgan damage scenario.

Beta-2-microglobulin is a well-known small protein expressed on the surface of nearly all nucleated cells and in most biological fluids. B2M plays an important role in antigen presentation, iron ion homoeostasis, and erythrocyte differentiation regulation. Abnormal forms of B2M are involved in amiloydosis, retinitis pigmentosa, immunodeficiency 43, multiple sclerosis, and thrombocytopenia. Beta-2-microglobulin has recently been proposed as a marker of worsening renal function and acute HF.[28,29] Beta-2-microglobulin is elevated in coronary artery disease and other forms of atherosclerosis and correlates with disease severity independently of other risk factors.[30,31]

Finally, IC1 is an alpha-globulin that regulates not only the complement system but also the plasma kallikrein–kinin, fibrinolytic, and coagulation systems. The biologic activities of IC1 can be divided into the regulation of vascular permeability and anti-inflammatory functions. IC1 is mainly present in lungs, liver, subcutaneous adipose tissue, and kidneys, and it has been shown to play a cardioprotective role after myocardial ischaemia and reperfusion.[32,33] Fattouch *et al.*[34] found that the inhibition of the classic complement pathway by IC1 appeared to be an effective mean of preserving ischaemic myocardium from reperfusion injury. This led to suggest that IC1 administration might be a rescue therapy in STEMI. Furthermore, Thielmann *et al.*[35] demonstrated that IC1 administration in emergency CABG with acute STEMI was safe and effective to inhibit complement activation and may reduce myocardial ischaemia–reperfusion injury. More recently, IC1 treatment has also been postulated to counteract myocardial infarction-induced inflammation in a rat model.[36] These data are consistent with our findings of increased IC1 levels among CS survivors. In another line of evidence, it has been reported that patients with more severe forms of septic shock could exhibit a relative

deficiency of IC1,[37] also in agreement with our findings in patients with CS.

Little is known about physiological factors affecting release variability of neither the identified proteins nor its kinetics in pathologic conditions. Beta-2-microglobulin and IC1 are not affected by the circadian rhythm. Actually, B2MG is used as a gold standard for reference genes that are stable and not affected by experimental conditions.[38] Fructose-bisphosphate aldolase B and L-FABP showed a weak circadian rhythm in murine models,[39] but no evidence reported in humans. Relative to fasting conditions, B2MG, ALDOB, and IC1 are not affected by fasting condition.[38] Liver-type fatty acid-binding protein was found regulated by fasting-feeding cycles in rats.[39] Lastly, relative to drug intake, ALDOB, L-FABP, and IC1 have no drug interaction. The only approved drug proven to interact with B2M is opium-derivatives. However, only a binding has been proposed, with no pharmacological action described.

Overall, these four proteins reflect multiorgan dysfunction, as well as systemic inflammation and immune activation.[23–37] During the early hours of CS, changes in the expression of these proteins may precede overt multiorgan failure and identify patients at a higher mortality risk. These data highlight the relevance of systemic involvement in CS, beyond primary pump failure. It may be speculated that the same pathways might be activated in the sickest patients suffering from non-cardiac shock.

This study is not without limitations. Both the Barcelona and CardShock cohorts were modestly sized but are in line with most CS literature on biomarkers. Indeed, CS cohorts with circulating sample biobanking are rare. Notably, both cohorts included very few patients with ventricular-assist devices (one in each cohort), and thus no subanalyses were performed in these patients. Although this may not correspond to the growing current clinical practice, it permits the analysis of the highest-risk patients when managed using conventional therapies, and thus may be useful for guiding the selection of candidates for advanced therapies. Finally, the sickest patients, those who die in the first hours, may be underrepresented due to the well-known difficulty of enrolment in critical situations. Future larger studies are needed to validate the scientific premise and estimate thresholds for clinical application.

Conclusion

Quantitative proteomics analyses in two independent cohorts of CS patients resulted in the definition of the CS4P risk assessment model, a circulating four-protein classifier (L-FABP, ALDOB, 2BMG, and IC1) that improves CS patient stratification according to short-term mortality risk. Additionally, the combination of the CS4P model to existing risk scores improved their overall performance predictive metrics, which may help clinicians in the early identification of high-risk CS patients for prompt invasive procedures, such as mechanical circulatory support.

Supplementary material

Supplementary material is available at *European Heart Journal* online.

Conflict of interest: F.R., E.S., E.B., C.G.-G., O.I.-E., and A.B.-G. have filed a patent concerning the use of CS4P for cardiogenic shock risk stratification. The other authors have nothing to declare.

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